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Assessment of Inter-alpha-trypsin heavy chain-4 peptides in differential diagnosis of ischemic and hemorrhagic stroke

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ABSTRACT:
Background: Differential diagnosis of Ischemic stroke (IS) and Hemorrhagic Stroke (HS) patient is crucial for augmenting the specific treatment. **Objective:** In the present study, we aimed to evaluate the role of the Inter-alpha-trypsin inhibitor heavy chain 4 (ITIH4) for the differential diagnosis of IS and HS patient. **Material and method:** This prospective study was carried out on 104 IS and 15 HS patients. Blood samples were collected at admission. Serum level of ITIH4 was estimated by indirect ELISA method using anti-ITIH4 peptide antibody (anti-ITIH4-peptide 1 to 9). Diagnosis sensitivity and specificity were determined by ROC curve analysis. **Results:** Out of panel of 9 antibodies, expression of Anti-ITIH4 peptide antibody 2 (p=0. 04), 5 (p=0.001), & 6 (p=0.0005) were significantly different between AIS and HS. Results shows sensitivity of 100%, 94%, 100% and specificity of 50% , 52% and 61% by anti-ITIH4 peptide antibody 2 (Cut off- >0.764; AUC- 0.720 ; 95% Cl 0.633-0.796), 5(Cut off- >0.774; AUC-0.779; 95% Cl 0.696-0.848) & 6 (Cut off- >0.738; AUC- 0.816; 95%Cl 0.737-0.879) respectively for differential diagnosis of AIS and HS.. **Conclusion:** Present study suggest that the Anti- ITIH4-peptide 2, 5, and 6 may be useful for differential diagnosis of IS and HS.
Key words: Acute Ischemic Stroke, Hemorrhagic Stroke, differential diagnosis, blood biomarkers

INTRODUCTION

Stroke remains to be a second most common cause of deaths each year and almost 1 in 8 (11.9%) deaths in worldwide. [1] According to stroke statistics 2016, the burden of disease (long-term disability and premature deaths) caused by stroke is set to be doubled worldwide by 2030. [2] In India 1.6 million cases of stroke were reported in 2015, at least one-third of whom were disabled.

Stroke is mainly categorized into two types i.e. Acute Ischemic (AIS) which accounts for 85% and Hemorrhagic stroke (HS), accounts for 15% of all strokes. Compared to AIS, HS is known to be associated with poor outcome with 30-day mortality rates of 37-52%.[3] Only 20% of HS survivors returns to their normal function at 6 months, these rates have not changed over the past 20 years. [4, 5]

Rapid recognition of AIS and HS is very crucial. CT scan and MRI are the only existing choice of test for differential diagnosis. [6, 7, 8] However CT is often normal after the onset of Ischemia and may remain normal in patients with mild Ischemic stroke. MRI is sensitive in detecting ischemia than CT. However, MRI has more contraindications, is more difficult to perform in ill patients, and takes longer than computed tomography scanning. [9] Other limitations associated with these techniques are high cost and nonavailability in most of the hospitals.

During the last decades, extensive research has been done on the discovery of a biomarker for differential diagnosis of AIS from HS. Biomarkers such as S100β, CRP, D-dimer, sRAGE, MMP9, and BNP were reported for differential diagnosis. [10, 11, 12] But only a few of them were used in routine diagnosis purpose. [13]

In our earlier studies, we have reported that serum level of Inter-α-trypsin inhibitor heavy chain 4 (ITIH4) proteins (120-kDa) could also be a useful biomarker for prognosis of AIS patients. [14, 15] In the present study, we aimed to evaluate the role of ITIH4 for differential diagnosis of AIS and HS.

MATERIALS AND METHODS

Ethical Statement

The study was approved by the Institutional Ethics Committee of Central India Institute of Medical Sciences (CIIMS) (No. CIIMS Res/ Stroke study /2011; dated 1/9/2011), Nagpur. Informed consents were taken from all the enrolled participants and their kin for the study.

Patients

The present study was carried out on a total of 119 subjects. All the subjects were selected from the

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inpatient department (IPD) wards and intensive care unit (ICU) of CIIMS Nagpur, India.

Diagnostic criterion
Diagnosis of AIS was based on the WHO definition of stroke, such as "rapidly developing signs of focal (or global) disturbance of cerebral function lasting >24 hrs (unless interrupted by surgery or death), with no apparent nonvascular cause, history, neurological examination and computerized tomography (CT)." Based on Neuroimaging data, the recruited participants were classified as AIS and HS. Similarly, a patient was confirmed for diabetes if he or she had a known history of type 2 diabetes mellitus (DM) and glucose levels were >110 mg/dL OR in a case of those without knowing DM, the admission blood glucose was ≥150 mg/dL (Figure 1).

Selected patients were subjected to the following protocol
Detailed history; CT scan within 12 h of admission to exclude non-AIS patients; the severity of stroke was evaluated using National Institute of Health Stroke Scale (NIHSS). 15 The NIHSS score consists of 15 items and a total score of 42 points. All patients were kept in the Intensive Care Unit (ICU) where the ambient temperature was maintained between 20 and 25 °C.

Inclusion and Exclusion criteria
Participants for the study were recruited by using a predefined exclusion and inclusion criteria. The distinction between AIS and HS was done on CT/MR scan observation made by the neurologist. An AIS was confirmed when an artery typically Middle Cerebral Artery (MCA), Posterior Cerebral Artery (PCA) or an Anterior cerebral artery (ACA) appears hyperdense indicative of a major occlusion of the vessel with thrombus formation, whereas hemorrhagic stroke was confirmed by the appearance of hyperdense to gray matter, and have a heterogeneous appearance in CT images. Confirm AIS and HS patients were included in the study. Patients with older age, i.e. age more than 85 years, Brain malignancy patients, Transient ischemic attack, brain operation, with severe systemic disease, dementia, psychiatric disease, active infection, refused to participate in the study, and who took discharge against medical advice were excluded from the study.

Blood Samples
Venous blood was collected from AIS patients at 0 hrs (i.e. at the time of admission) and at the time of discharge or death of AIS patient. The last blood samples drawn before the patient expired were considered as an expired time sample. All the blood was allowed to clot and after centrifugation (100 ×g for10 min); serum was separated and stored at -20 °C until it was used.

DESIGNING AND SYNTHESIS OF PEPTIDES AND ANTI-PEPTIDE OF ITIH4
The antigenic peptides of ITIH4 (Panel I) were determined by Kolaskar and Tongaonkar (1990) method by using online software "Molecular Immunology Foundation-Bioinformatics software (MIF-Bioinformatics software)" (Panel II). Total thirty-eight antigenic sequences were identified in ITIH4 using MIF Bioinformatics software. Multiple sequence alignment of these antigenic sequences was done using National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) to obtain the sequence similarities with other non-redundant protein database sequences. After blast analysis 12 antigenic sequences were found to be specific to ITIH4. These peptide sequences were then checked for suitability for antibody production using the Innovagen software. Only nine peptide sequences were found to be suitable for antibody production (Panel III). Accordingly, nine anti-peptides were synthesized by GenicBio lab, Shanghai, China.

a) >gi|219517748|gb|AAI36393.1| ITIH4 protein [Homo sapiens]

MKPPRPVRTCSKVLVLLSLLAIHQTTTAEKNGIDIYSLTVDSRVSSRFAHT
VVTSRVVNRANTVQEATFQMELPKKAFITNFSMIIDGMTYPGIIKEKAEA
QAQYSAAVAKGKSAGLVKATGRNMEQFQVSVSVAPNAKITFELVYEELL
KRRLGVYELLLKVRPQQLVKHLQMDIHIFEPQGISFLETESTFMTNQLVD
ALTTWQNKTKAHIRFKPTLSQQQKSPEQQETVLDGNLIIRYDVDRAISGG
SIQIENGYFVHYFAPEGLTTMPKNVVFVIDKSGSMSGRKIQQTREALIKIL
DDLSPRDQFNLIVFSTEATQWRPSLVPASAENVNKARSFAAGIQALGGTN
INDAMLMAVQLLDSSNQEERLPEGSVSLIILLTDGDPTVGETNPRSIQNN
VREAVSGRYSLFCLGFGFDVSYAFLEKLALDNGGLARRIHEDSDSALQL
QDFYQEVANPLLTAVTFEYPSNAVEEVTQNNFRLLFKGSEMVVAGKLQD
RGPDVLTATVSGKLPTQNITFQTESSVAEQEAEFQSPKYIFHNFMERLWA
YLTIQQLLEQTVSASDADQQALRNQALNLSLAYSFVTPLTSMVVTKPDD
QEQSQVAEKPMEGESRNRNVHSGSTFFKYYLQGAKIPKPGLDHTEASFS
PRRGWNRQAGAAGSRMNFRPGVLSSRQLGLPGPPDVPDHAAYHPFRRL
AILPASAPPATSNPDPAVSRVMNMKIEETTMTTQTPAPIQAPSAILPLPGQS
VERLCVDPRHRQGPVNLLSDPEQGVEVTGQYEREKAGFSWIEVTFKNPL
VWVHASPEHVVVTRNRRSSAYKWKETLFSVMPGLKMTMDKTGLLLLS
DPDKVTIGLLFWDGRGEGLRLLLRDTDRFSSHVGGTLGQFYQEVLWGSP
AASDDGRRTLRVQGNDHSATRERRLDYQEGPPGVEISCWSVEL

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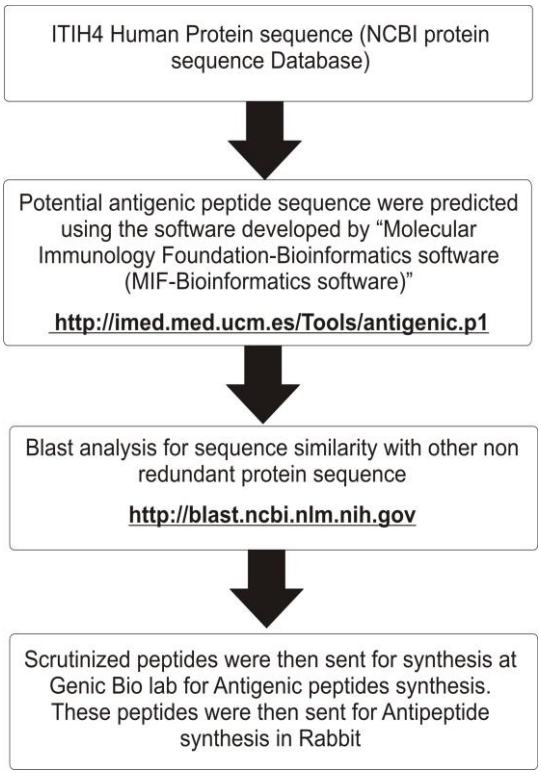
Panel I: Reference sequence of human ITIH4 obtained from National Center for Biotechnology Information (NCBI) Protein reference sequence database



Panel II: Detailed flow chart of the steps followed for synthesis of antigenic peptides of ITIH4 and production anti-ITIH4-peptide antibodies

Panel III: List of nine antipeptides antibodies generated against the selected peptides of ITIH4

Sr. No.	Antibody	Sequence
1	Anti ITIH-4 P1	LLLKVRPQQLVKH-C
2	Anti ITIH-4 P2	REALIKILDD-C
3	Anti ITIH-4 P3	PEGSVSLIILLT-C
4	Anti ITIH-4 P4	RYSLFCLGFGFDVSY-C
5	Anti ITIH-4 P5	C-GPDVLTATVSGK
6	Anti ITIH-4 P6	C-LNLSLAYSFV
7	Anti ITIH-4 P7	C-TFFKYYLQGAKIPKPEA
8	Anti ITIH-4 P8	C-LLLSDPDKVT
9	Anti ITIH-4 P9	C-LGQFYQEVLWG

Note- P- Peptide

ITIH4 estimation using anti-ITIH4 peptide antibody

ITIH4 was estimated using in-house developed indirect enzyme-linked Immunosorbent assays (ELISA). In brief, Microtiter ELISA wells were coated with 100 µl (1:400) of serum samples taken from AIS patients and blocked with 200 µl of 2.5% BSA in phosphate buffer saline (PBS) for 2hrs. After washing with PBS, the polyclonal antibody against ITIH4 peptides was added (GenicBio Lab, Shanghai, China), and plates were incubated at 37 °C for 45 min. The wells were again washed, followed by addition of secondary antibody (goat anti-rabbit immunoglobulin horseradish peroxidase; IgG-HRP; (Merck Millipore, India)) and incubated for 45 min at 37 °C. After another wash, antibody reactivity was detected via the addition of 100 µl Tetramethylbenzidine hydrogen peroxide (TMB/H2O2) substrate solution to the wells, which were then incubated at room temperature for about 5 min. The reaction was stopped with 100 µl of 2.5 N H2SO4, and the absorbance of each well was read by Readwell Touch - Automatic Elisa Plate Reader (Robonik India Pvt. Ltd) at 450 nm.

STATISTICAL ANALYSIS

All the statistical analysis was performed using the MedCalc software version 10. Baseline characteristics were compared using a chi-square test. Similarly, the ITIH4 peptide level in AIS and HS patients were compared using the student t-test. Similarly, Receiver operator characteristic (ROC) curve analysis was done to calculate the sensitivity and specificity of each peptide. $P<0.05$ were considered as significant for the entire test.

RESULTS

A total of 119 consecutive patients admitted to Neurology and Neurosurgery Intensive Care Unit during the period of 2011-2014 were included in the present study. 104 (87.4%) patients with Ischemic stroke (73 males & 31 females) and 15 (12.6%) patients with Hemorrhagic stroke (13 males & 2 females) were classified on the basis of CT scan and MRI report performed in the Emergency Department. Baseline characteristics associated with AIS and HS patients are given in table 1. We found that 66% of AIS and HS patients were older age group, i.e. above 50 years with a higher proportion of males [86, (72%)]. Hypertension was found to be a major risk factor present in AIS [70, (67%)] and HS [10, (67%)]. Similarly, Cardiac disease was found to be significantly associated with HS compared to AIS. ITIH4 level in the serum samples were estimated by

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indigenous ELISA developed using panels of anti-ITIH4 peptide antibody (n=9) produced against the synthetic peptides of ITIH4

Table 1: Baseline clinical characteristics of AIS and HS patients

Baseline Characteristics	**Total n (%)**	**AIS Patients (n=104)**	**HS Patients (n=15)**
Age			
≤ 50 years	40 (34)	38 (37)	2 (13)
> 50 years	79 (66)	66 (63)	13 (87)
Sex			
Male	86 (72)	73 (70)	13 (87)
Female	33 (28)	31 (30)	2 (13)
Associated risk factor			
Hypertensive	80 (67)	70 (67)	10 (67)
Diabetes	32 (27)	29 (28)	3 (20)
Ischemic heart diseases	8 (7)	5 (5)	3 (20)
Cardiac disease	7 (6)	3 (3)	4 (27)*
Other disorders	33 (28)	27 (26)	6 (40)
Past History of Stroke	7 (6)	6 (6)	1 (7)
Behavioral factors			
History of smoking	8 (7)	8 (8)	0 (0)
History of alcohol	12 (10)	11 (11)	1 (7)
Stroke Therapy			
Thrombolysis	11 (9)	10 (10)	1 (7)
Decompression surgery	3 (3)	3 (3)	0 (0)

*Note - *p value <0.05*

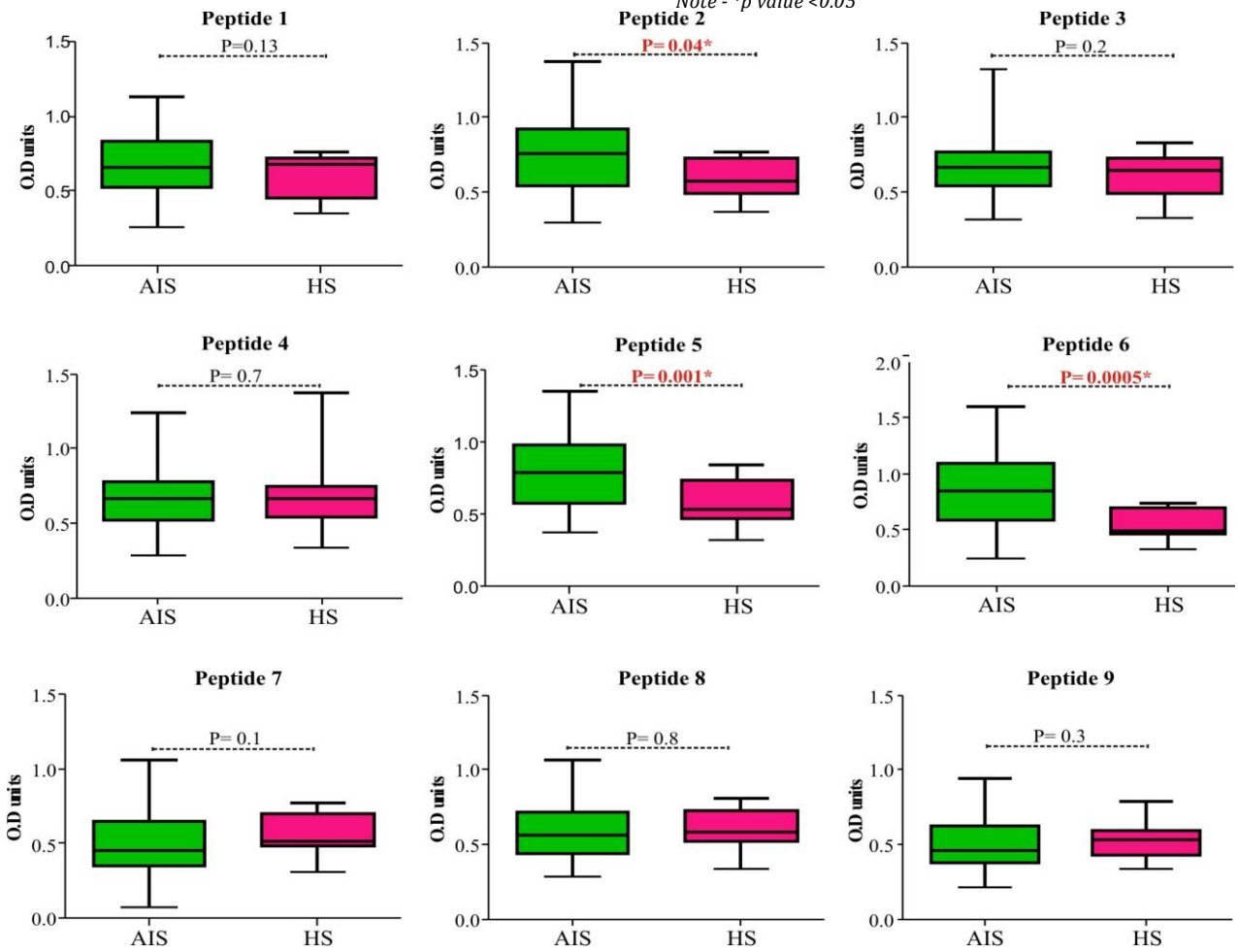

Fig 1: ITIH4 estimation in the serum samples of acute ischemic stroke (AIS) and hemorrhagic stroke (HS) patients.

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Figure 1 & 2 depicts the results of ITIH4 estimation. Out of panel of 9 antibodies, expression of Anti-ITIH4 peptide antibody 2 (p=0. 04), 5 (p=0.001), & 6 (p=0.0005) were significantly different between AIS and HS.

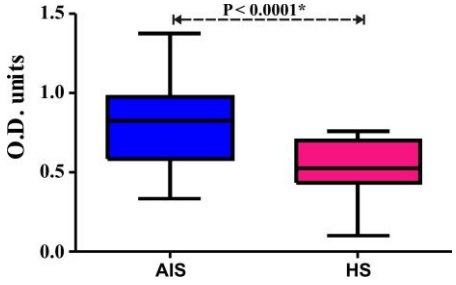
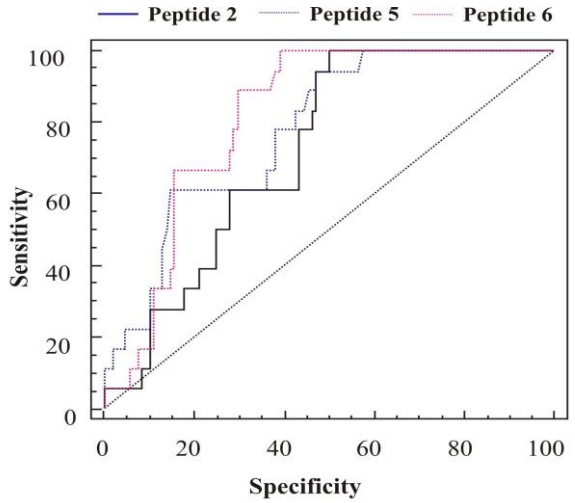


Fig 2: ITIH4 Mean peptide level in ischemic stroke (AIS) and hemorrhagic stroke (HS) patients

Figure 3 Depicts the results of the ROC curves analysis of anti-ITIH4 peptide antibody 2, 5 & 6 for differential diagnosis of AIS and HS. Results shows sensitivity of 100%, 94%, 100% and specificity of 50%, 52% and 61% by anti-ITIH4 peptide antibody 2 (Cut off- >0.764; AUC- 0.720; 95% Cl 0.633-0.796), 5(Cut off- >0.774; AUC-0.779; 95% Cl 0.696-0.848) & 6 (Cut off- >0.738; AUC- 0.816; 95%Cl 0.737-0.879) respectively for differential diagnosis of AIS and HS.



Peptide	Cut off	Sensitivity	Specificity	p value	AUC	95% confidence interval
ITIH4 P2	>0.764	100	50	0.0001*	0.720	0.633 -0.796
ITIH4 P5	>0.774	94	52	0.0001*	0.779	0.696 -0.848
ITIH4 P6	>0.738	100	61	0.0001*	0.816	0.737 -0.879

Fig 3: Comparative analysis of Receptor Operating Curve (ROC) showing cut off value, sensitivity and specificity of anti-ITIH4-peptide antibody 2, 5 and 6

DISCUSSION

In the present study, we evaluated the role of ITIH4 for differential diagnosis of AIS and HS. We found that out of panel of 9 antibodies, expression of Anti-ITIH4 peptide antibody 2, 5, & 6 were significantly different between AIS and HS.

Mortality and morbidly in stroke is highly depends on the early diagnosis of Ischemic or Hemorrhagic stroke and applying the efficient treatment in a timely manner. [16] In emergency setting, diagnosis of stroke relies on historical data, neurologic examination, and neuroimaging techniques, including brain computerized tomography (CT) and magnetic resonance imaging (MRI) scans. [17] However, the diagnosis of Ischemic or Hemorrhagic stroke within the first hour is not always straightforward. CT is often normal after the onset of ischemia and may be normal in mild stroke cases; on the contrary, MRI may not be feasible in acutely ill most of the hospitals does not advanced specialized MRI services. [18] Thus a lack of early diagnostic tools delays the treatment of patients who is otherwise eligible for treatment with intravenous thrombolysis. [19, 20, 21] Therefore a reliable and adjunct biomarker can easily measure in blood would be invaluable for cost-effective diagnosis of Ischemic and Hemorrhagic stroke.

Recently studies related to some markers who are thought to be efficient in establishing the diagnosis of ischemic from hemorrhagic stroke are being carried out. Considerable studies has been done on brain-specific protein biomarker such as S100 calcium-binding protein B (S100B), glial fibrillary acidic protein (GFAP)] and neuronal cells [e.g., ubiquitin C-terminal hydrolase-L1 (UCH-L1), neuron-specific enolase (NSE), αII-spectrin breakdown products SBDP120, SBDP145, and SBDP150, myelin basic protein (MBP), neurofilament light chain (NF-L), tau protein, visinin-like protein-1 (VLP 1), NR2 peptide that could have been used for diagnosis and prognosis of stroke.[22,23] The study reported the plasma S100β concentration in the ICH group was significantly higher than in the IS group. In the ICH group, the plasma S100β concentration was significantly elevated in patients with poor functional outcome vs. those with the favorable functional outcome.[24] Similarly, MMP-9 and D-dimer were found to be effective separately at a differential diagnosis of ischemic stroke hemorrhagic stroke ($p<0.05$).[25] A subsequent

multicenter study of S100B, NSE, GFAP, and activated protein C-protein C inhibitor complex (APC-PCI) demonstrated a significant ability of GFAP to distinguish ICH from ischemic stroke.[26,27].

Inter-alpha-trypsin-inhibitor heavy chain-4 (ITIH4) protein is a novel marker for Acute Ischemic Stroke. This 120-kDa ITIH4 protein showed high expression in control, but no expression or barely expressed in patients with AIS, and then the protein return to normal level in serum gradually as the patient was getting better. [14] We have also reported the Expression of S-100ββ, Neuron-specific enolase (NSE), (Interleukin) IL-2, and IL-10 was highly correlated with ITIH4 expression. [28] Since ITIH4 is a type II Acute Phase protein (APP), involved in the inflammatory response to trauma [29] we also think that it may have a distinct role in the pathophysiology of Ischemic and Hemorrhagic stroke which could be used for differentiation. In the present study, we found that peptide 2, 5 and 6 out of 9 peptides showed an increase in expression of ITIH4 in the serum sample of AIS patients compared to HS. This shows that peptide 2, 5 and 6 are the only immunodominant and specific peptide sequence of ITIH4. Therefore, the estimation of serum ITIH4 by antibody specific to peptides 2, 5 and 6 can be an important marker for differential diagnosis of Ischemic from Hemorrhagic stroke.
Despite some interesting finding, our study associated with major limitations such as less number of samples. However, the findings obtained were very encouraging to conduct a large-scale study.

CONCLUSIONS

Based on the results it can be concluded that estimation of serum ITIH4 peptide level may be helpful as an adjunct biomarker for differential dignosis of AIS and HS.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest for this article.

SOURCE OF FUNDING

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Ref. No. CIIMS/Res/DBT-Strok/2010 Date 4/7/2010

The collection of blood, CSF and other body fluids of diseased and healthy control groups for experimental analysis in the DBT project titled "**Development of low cost prognostic kit (s) for brain stroke patients**" have been approved by Institutional Ethics Committee of Central India Institute of Medical Sciences (CIIMS), Nagpur.

Members of Institutional Ethical Committee(IEC)

Name	Qualification	Position held in the IEC	Signature
Dr. J. Y. Deopujari	MD, PhD (Ayurveda) Ayurvedic Physician, Nagpur	Chairman, IEC	
Dr. G. M. Taori	MD, FRCP (C), Director, CIIMS	Secretary, IEC	
Dr. H. F. Daginawala	M.Sc, PhD, FMASc Senior Research Consultant, CIIMS	Member, IEC	
Mr. N. S. Bhattad	LLB, Advocate, Nagpur Chairman, Legal Advisory Committee, CIIMS	Member, IEC	
Mr. R. S. Bhattad	Chartered Accountant, Nagpur. Chairman, Management Committee, CIIMS	Member, IEC	
Mrs. Nandini Deshmukh	M.A.,M.S.W Social Worker, Nagpur	Member, IEC	
Dr. H. J. Purohit	M.Sc, PhD Sr. Scientist and Head-Environmental Genomics Unit, NEERI, Nagpur	Member, IEC	

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Research Centre
रिसर्च सेंटर
Consent Form
अनुमति पत्र

मरीज का नाम : _____

Name of the Patient : _____

उम्र : _____ लिंग : _____ ओ.पी. नं. _____ आय.पी. नं. _____

Age : _____ Sex : _____ OP No. : _____ IP No. _____

भरती का दिनांक _____

Date of Admission _____

मै, मरीज _____ उम्र _____ और, या

मैं _____ (पिता/भाई/पति/पत्नी/पुत्र/पुत्री _____),
मरीज के रोग निदान एवं चिकित्सा या संशोधन कार्य के लिए, द्रव्य पदार्थ या माँसपेशियों को निकालने के लिए अनुमती प्रदान करता / करती हूँ, जो आपके संशोधन कार्य के लिए भी काम आ सकेगा।

मरीज का द्रव्य पदार्थ या माँसपेशियों का नमूना इसी तरह अन्य संशोधन शाला में भी उपलब्ध कराया जा सकता है, जिससे मिलने वाली राशी का उपयोग नमूनों को बेहतर स्थिती में रखने एवं ससंच्यित करने के लिए किया जाएगा।

I, Patient _____ Age _____ and/or

I, _____ (father/brother/Wife/
son/daughter, do not have any objection for any body fluids or tissue taken for diagnositic and or therapeutic prutposes, to be utilised for the research purpose, and can be supplied to other Research Institutions on payment basis. The amount received will be utilized only for processing and storage of such materials.

Signature / Thumb impression of the patient or patient relative _____

मरीज या मरीज के रिश्तेदार का हस्ताक्षर या अंगूठा _____

Name _____

नाम _____

Relationship _____

(रिश्ता) _____

Signature / Thumb Impression : Witness _____

हस्ताक्षर / अंगूठा : साक्षीदार _____

Date _____

दिनांक _____

Name _____

नाम _____